Development of an *in situ* Hybridization Method for Neurohypophysial Hormone mRNAs Using Synthetic Oligonucleotide Probes

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ABSTRACT—Vasopressin (AVP) and oxytocin (OXT) mRNAs are highly homologous. We developed an *in situ* hybridization method to discriminate the AVP and the OXT mRNAs using synthetic 22mer deoxyoligonucleotides as probes which have several advantages over the use of cDNAs, e.g., highly specific, easy to obtain a designed probe, and easily accessible to cellular mRNAs. The probes were radiolabeled at the 5′ ends with ^{32}P, applied to rehydrated paraffin sections of rat and/or toad hypothalami, and were visualized by autoradiography. RNase treatment before incubation with the probes and measurement of melting temperature showed that the probes actually paired with tissue RNAs. The specificity of hybridization signals was checked by the following tests: absorption test, competition test, a use of alternate probes complementary to the different regions of the same mRNA, cross species hybridization, and comparisons with the immunohistochemical localization of AVP and OXT in adjacent or the same tissue sections. These tests showed that the oligonucleotide probes specifically discriminate the AVP mRNA from the highly homologous OXT mRNA. Furthermore, cross species hybridization clarified that an oligonucleotide probe can discriminate nucleotide sequences which include 2 mismatching bases. The use of multiple probes complementary to different loci in the same mRNA showed not only the specificities of the hybridization signals, but also its usefulness to enhance hybridization signals.

INTRODUCTION

Arginine vasopressin (AVP) and oxytocin (OXT) are mammalian neurohypophysial hormones produced mainly in magnocellular neurons in the supraoptic (SON) and the paraventricular nuclei (PVN). They are released from neurosecretory terminals into blood capillaries in the neurohypophysis, and play important physiological roles, e.g., regulation of plasma osmolarity and blood pressure by AVP, and oxytocic action and stimulation of milk ejection by OXT. It is therefore important to examine expressions of AVP and OXT genes in magnocellular neurons in various physiological statuses. A recently developed *in situ* hybridization (ISH) method is the most plausible candidate for this examination.

The structures of rat AVP and OXT genes recently clarified [1] show that the AVP mRNA and the OXT mRNA share an extremely homologous region, the exon B, the homology of which is about 95 %. Since cDNAs can hybridize with mRNAs the homology of which is approximately 65 % [2], the presence of the AVP mRNA has been detected with a spliced cDNA probe complementary to the glycoprotein encoding region which is not present in the OXT mRNA [3–7], while the OXT mRNA has been localized with a probe complementary to the 3′-end of neurophysin (NP) and the 3′-untranslated region [3]. A problem arising here is the occurrence of vasotocin (AVT) especially in fetal brains of mammals [8, 9]. A possibility that the cDNA probes hybridize with the AVT mRNA makes it difficult to apply the ISH method in a study of ontogeny of the

neurosecretory system. Moreover, the use of cDNA probes entirely depends on their availability that requires facilities for recombinant DNA techniques. One of possible ways to overcome these problems is the use of synthetic deoxyoligonucleotides as probes for ISH, the technique developed in our laboratory [10–12]. The use of oligonucleotides as probes for ISH further can have several advantages over the use of cDNAs, that is, highly specific [2, 13, 14], easy to obtain a designed probe, easy to prepare and to label in an ordinary laboratory [15] and easily accessible to cellular mRNAs [16–18].

We designed 22mer oligonucleotide probes to discriminate localization of AVP and OXT mRNAs in paraffin sections. We further revised our previous ISH protocol [10, 11] by checking each staining step. A fixative solution was also carefully screened to stain the same or adjacent tissue sections by both ISH and immunohistochemical methods, because demonstration of AVP and OXT is crucial for better understanding of their gene expressions. Specificity of the present method was confirmed by various tests including cross species hybridization with the mRNAs of toad neurohypophysial hormones, nucleotide sequences of which were recently determined by Nojiri *et al.* [19]. Through the specificity tests, we tried to elucidate technical limitations of the present method and to confirm its general applicability in gene expression studies of many other peptides and proteinaceous hormones.

MATERIALS AND METHODS

Preparation of tissue sections

Male Wistar-Imamichi rats (6–8 weeks old) and adult Japanese toads of both sexes captured in the autumn were obtained from commercial sources. They were killed by decapitation, and the hypothalami and the pituitaries were rapidly taken out and immersed in fixative solutions at 4°C for 2 days. Since a preliminary experiment showed that fixation by perfusion markedly decreased hybridization signals, we preferred fixation of tissues by immersion. Fixatives tested were: Bouin's solution, modified Bouin's solution which does not

include acetic acid, 4% paraformaldehyde (PFA) in 0.05 M phosphate buffer (pH 7.3), a buffered solution containing 2% PFA and 1% glutaraldehyde (GLA), and that including 2% PFA, 1% GLA and 1% picric acid (PA). As is described in the Results section, the mixture of PFA, GLA and PA (PGP solution) yielded satisfactory results in both ISH and immunohistochemical staining among these fixatives. Therefore, the PGP solution was routinely used in the present study.

After fixation, the hypothalami were washed in 70% ethanol at 4°C for 24 hr twice. They were then dehydrated through graded ethanols, and were embedded in paraplast. Serial transverse sections were cut at 8 or 10 μm, separated into several groups, and were mounted on gelatinized slides. Some hypothalamic tissues were washed in cold 0.05 M phosphate buffer (pH 7.3) after fixation, rapidly frozen in butanol cooled in dry ice-acetone, and were cut at 20 μm on a frozen microtome. They were also mounted on gelatinized slides.

Preparation of synthetic oligonucleotide probes

Four 22 mer oligonucleotide probes (Fig. 1) were synthesized by the phosphoramidite method [20] and were purified by polyacrylamide gel electrophoresis. They are complementary to the regions in the rat AVP mRNA encoding AVP (2–9) and AVP–NP (1–8), to that in the rat OXT mRNA encoding OXT–NP (1–8), and to that in the toad AVT mRNA encoding AVT (−1 to 7). They are thus referred to as AVP, AVP–NP, OXT–NP and AVT-OXT probes, respectively. The nucleotide sequence of AVT/OXT probe is exactly complementary to the corresponding region of OXT mRNA, while the nucleotide at position 9 of this probe is mismatched with the counterpart of AVP mRNA (i.e., 95% homology). The AVP probe has 2 mismatching positions with the AVT mRNA (91% homology), and 6 mismatching positions with the OXT mRNA (73% homology). The AVP–NP and OXT–NP probes differ at 10 positions (55% homology). The homology of the corresponding region of toad mesotocin mRNA with the AVT–OXT probe is 77%, and that with the AVP probe is 73%.

The probes were labeled at the 5' ends with T4

AVP mRNA | Sig | AVP | AVP-NP | GP |

AVP(2-9) AVP-NP(1-8)

```
            Cys Tyr Phe Gln Asn Cys Pro Arg Gly
AVP
AVP mRNA   5' UGC UAC UUC CAG AAC UGC CCA AGA GGA 3'
Probe         3' G AAG GTC TTG ACG GGT TCT CCT 5'
Template      AC TTC CAG AAC TGC

            Ala Tyr Ser Asp Met Glu Leu Arg
AVP-NP
AVP-NP mRNA  GCC ACA UCC GAC AUG GAG CUG AGA
Probe        GG TGT AGG CTG TAC CTC GAC TC
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OXT mRNA | Sig | OXT | OXT-NP |

OXT-NP(1-8)

```
            Ala Ala Leu Asp Leu Asp Met Arg
OXT-NP
OXT-NP mRNA  GCU GCG CUA GAC CUG GAU AUG CGC
Probe        GA CGC GAT CTG GAC CTA TAC GC
```

AVT mRNA | Sig | AVT | AVT-NP | GP |

AVT((-1)-7)

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            Ala Cys Tyr Ile Gln Asn Cys Pro
AVT
AVT mRNA     GCC UGC UAC AUC CAG AAC UGC CCC
Probe        GG ACG ATG TAG GTC TTG ACG GG
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Fig. 1. Design of synthetic oligonucleotide probes in the present experiments. Nucleotide sequences of the probes are shown with those of the complementary mRNAs. Amino acid sequences encoded by the mRNA sequences are also shown. Numbers in parentheses indicate amino acid positions. Sig, signal peptide; NP, neurophysin; GP, glycoprotein.

polynucleotide kinase using $[\gamma-^{32}P]$ ATP to a final specific activity of $4-6 \times 10^7$ cpm/μg by the procedure of Maxam and Gilbert [15]. Radioactivity of the labeled probe was measured by liquid scintillation counting of Cerenkov radiation.

Procedure for in situ hybridization

After rehydration, tissue sections were treated with proteinase K (1 μg/ml; Sigma, type XI) in 0.1 M Tris buffer (pH 8.0) containing 50 mM EDTA at 37°C for 30 min, and were briefly washed in doubly deionized water at room temperature. They were then rinsed in $2 \times$ SSC ($1 \times$ SSC contains 0.15 M NaCl and 0.015 M sodium citrate), preincubated in a hybridization buffer (0.9 M NaCl, 6 mM EDTA, 0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinylpyrrolidone and 100 μg/ml denatured salmon sperm DNA in 90 mM Tris buffer, pH 7.5) at room temperature for 1 hr, and were placed in a moist chamber. The radiolabeled oligonucleotide probe was diluted to 1×10^4 cpm/μl in hybridization buffer, and 80 μl of the probe solution was applied to each slide glass. Sections were coverslipped, and were incubated at 30°C overnight. After removing coverslips in cold

$6 \times$ SSC, the sections were washed in $6 \times$ SSC firstly at 4°C for 10 min, then at about 20°C for 20 min twice, and again at 4°C for 10 min. The sections were then dehydrated through graded ethanols (70, 90 and 100%) containing 0.3 M ammonium acetate and were air-dried. Thereafter, the sections were dipped in Sakura NR–M2 emulsion diluted 3:2 with 0.3 M ammonium acetate, air-dried for 30 min, and were exposed for 1 to 3 weeks. After development in Kodak D–19 and fixation, they were dehydrated and were coverslipped with Permount (Fisher).

Methodological checks

Proteinase treatment The proteinase treatment after rehydration has been considered to increase accessibility of the probes to tissue mRNAs. We examined whether the proteinase treatment actually increase hybridization signals in the rat hypothalamic sections fixed by PFA only and those fixed by the PGP solution.

Acetylation Acetylation of tissue sections was reported to decrease non-specific binding of probes, so that background could be reduced [21]. Therefore, proteinase treated rat tissue sections were immersed in freshly prepared 0.25 % acetic anhydride in 0.1 M triethanolamine buffer (pH 8.0) for 10 min prior to preincubation.

Effect of long-term storage of tissue sections Tissue sections from the same rats were separated into several groups, and were left unhydrated. They were kept in a desiccated box at a cool place. A group of tissue sections were periodically taken out, and the AVP mRNA was stained by ISH method during a period of more than 18 months.

Concentration of labeled probes Hybridization mediums containing different levels of probe concentrations between 5×10^3 cpm/μl to 2×10^4 cpm/μl were prepared, and were applied to tissue sections to determine an appropriate probe concentration.

Specificity tests for ISH

RNase pretreatment Sections treated with proteinase were incubated with ribonuclease (100 μg/ml; BDH Chemicals) in 0.1 M Tris-HCl (pH 7.5) at room temperature for 1 hr, and were washed in doubly deionized water. The sections

were then hybridized with labeled AVP–NP probe.

Estimation of melting temperature (Tm)
When a probe molecule is paired with the complementary mRNA region by hydrogen bonds, the Tm value experimentally determined was similar to those empirically determined and theoretically calculated [22]. As for oligonucleotide probes of around 20 bases, empirical Tm values for filter hybridization were between 50–60°C. An experimental Tm value was determined by modifying the washing procedure after incubation with the probes, that is, tissue sections were washed $6 \times SSC$ at a series of graded temperature (18–70°C) for 20 min after rinse in cold $6 \times SSC$. The AVT/OXT probe was used in this experiment. In the hybridized rat sections, a $100 \mu m \times 100 \mu m$ square was settled in the OXT region of the PVN. The specific numbers of silver grains within the squares were determined, and were plotted to estimate graphically the Tm value after the logit transformation.

Absorption test   A 14mer template oligonucleotide complementary to the AVP probe (Fig. 1) was synthesized, and a 20-fold amount was added to a hybridization medium so as to absorb the probe. Rat hypothalamic sections were incubated in this absorbed hybridization medium.

Competition test   Rat hypothalamic sections were incubated in a hybridization medium containing the labeled AVP–NP probe and a 10-fold amount of unlabeled AVP–NP probe. A similar experiment was performed also for the OXT–NP probe. As the control of these competition tests, the unlabeled mismatching probes were added to the hybridization mediums, e.g., the unlabeled OXT–NP probe to the labeled AVP–NP probe and *vice versa*.

Use of different probes to the same mRNA
The AVP and AVP–NP probes were complementary to different regions in the same mRNA. The localization of the AVP probe was thus compared with that of the AVP–NP probe. In addition, the same amounts of labeled AVP and AVP–NP probes were mixed so as to keep the radioactivity of incubation medium at 1×10^4 cpm/μl, and were applied to rat hypothalamic sections.

Cross species hybridization   The limitation of the oligonucleotide probes to discriminate mismatching sequences was examined by using naturally occurring homologues of mRNAs of neurohypophysial hormones in the rat and the toad hypothalami. The AVP probe was applied to sections of the toad hypothalamus, and the resulting hybridization signals were compared to those obtained by use of the AVT/OXT probe. Meanwhile, the AVT/OXT probe was applied to sections of the rat hypothalamus.

Correspondence to immunohistochemical localization of neurohypophysial hormones   The distributions of hybridization signals were compared with immunohistochemical localization of neurohypophysial hormones in the same or adjacent sections of the rat and toad hypothalami. For precise comparison, pairs of mirror image sections of the rat hypothalamus were utilized.

Immunohistochemistry

Tissue sections for immunohistochemistry were stained by the avidin-biotin-peroxidase complex (ABC) method using Vectastain ABC kit (Vector), the procedure of which was described elsewhere [11]. In the present study, rabbit anti-AVP (Bioproducts, batch #001) was diluted 1:32,000 with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin; and rabbit anti-OXT (a gift from Professor S. Kawashima, Hiroshima University) was diluted 1:20,000 with PBS. Since the anti-AVP antiserum cross-reacts completely with AVT, toad hypothalamic sections were stained with this antiserum as was described previously [23].

ISH and immunohistochemistry double staining
Tissue sections were first stained immunofluorescently with fluorescein-labeled avidin D (Vector; diluted 1:250 with bicarbonate-buffered saline, pH 8.2) that was replaced with ABC, photographed with a fluorescence microscope, and were processed for ISH. The use of an IgG-fractionated antiserum was required for this procedure. Otherwise, intensity of hybridization signals was markedly reduced probably by degradation of mRNAs by RNase in the serum.

Specificity tests of immunohistochemistry   In addition to the specificity tests previously de-

scribed [11], tissue sections were stained with AVP and OXT antisera preabsorbed with antigen conjugated CNBr–Sepharose 4B (Pharmacia) columns. These tests confirmed the specificity of immunohistochemical stainings in the present study.

RESULTS

Autoradiographic silver grains that represent hybridization signals of the AVP, AVP–NP and OXT–NP probes were localized densely over the magnocellular neurons of the SON, the PVN, the circular nucleus, the anterior commissural nucleus (ACN) and other accessory magnocellular nuclei in the rat hypothalamus (Figs. 2 and 3). The localization of AVP probe coincided with that of AVP–NP probe, while that of OXT–NP probe showed an independent pattern. The localization of hybridization signals was consistent with the immunohistochemical distribution of corresponding neurohypophysial hormones (Figs. 2 and 3). It was also true in the toad hypothalamus in which the AVT/OXT probe showed similar distribution to immunoreactive (ir) AVT in the magnocellular part of the preoptic nucleus (Fig. 5). In the rat hypothalamus, magnocellular neurons in the ven-
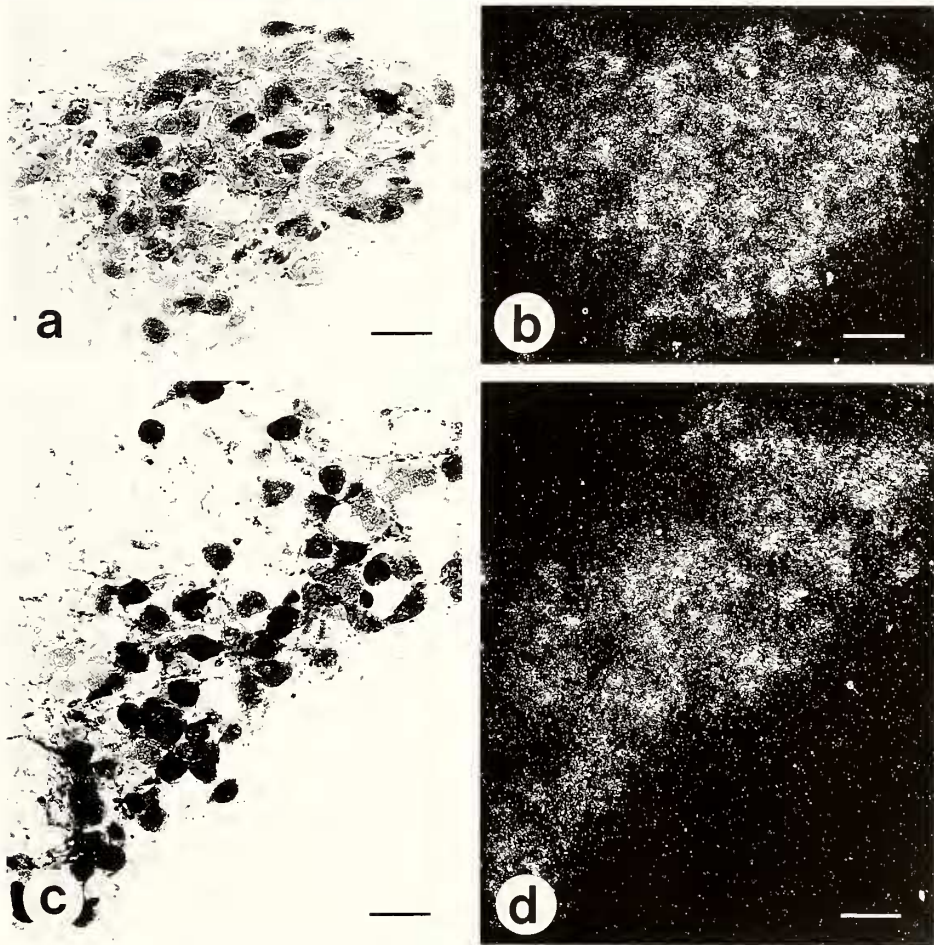

FIG. 2. Immunoreactive (ir) AVP (a) and OXT (c) neurons and hybridization signals of the AVP mRNA (b) and the OXT mRNA (d) in the paraventricular nucleus. Note parallel distribution of autoradiographic signals for the AVP mRNA (b) to AVP-ir neurons in mirror image section (a, counterstained with cresyl violet). Distribution of signals for the OXT mRNA (d) is also parallel to OXT-ir neurons in the adjacent section (c). Scale bar, 50 μm.
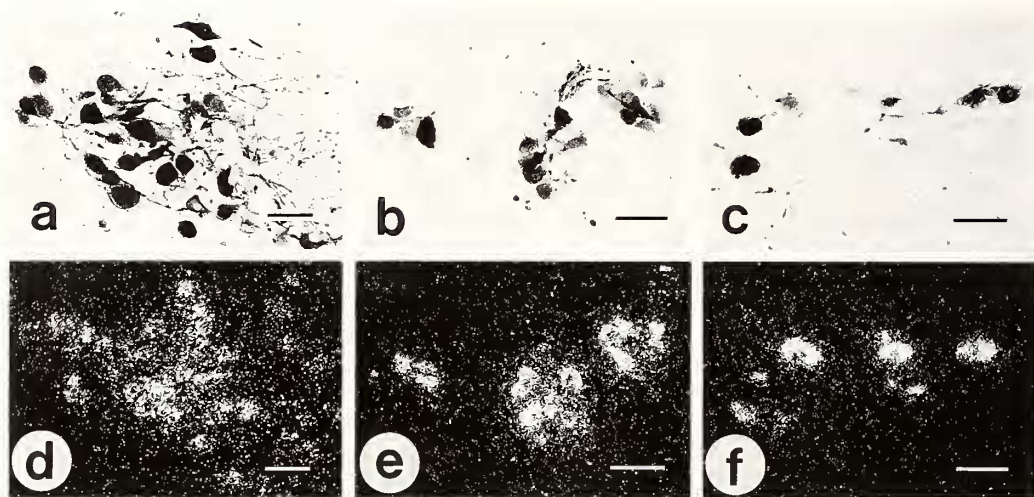
Fig. 3. Immunoreactive (ir) OXT (a) and AVP (b, c) neurons and hybridization signals of the OXT mRNA (d) and the AVP mRNA (e, f) in the anterior commissure nucleus (a, d), the circular nucleus (b, e), and the fornical nucleus (c, f). Autoradiographic signals for the OXT mRNA (d) are distributed parallel to OXT-ir neurons in the adjacent section (a). Signals for the AVP mRNA (e, f) are also distributed parallel to AVP-ir neurons in mirror image sections (b, c, counterstained with cresyl violet). Scale bar, 50 μm.

tral region of the SON and the dorsolateral region of the PVN were mainly AVP-ir, coinciding well with the localization of the AVP and AVP–NP probes. While the dorsal region of the SON, the ventromedial region of the PVN and the ACN were composed of OXT-ir neurons, and the OXT–NP probe was localized in these regions (Figs. 2 and 3). However, noticeable hybridization signals were not detected in the suprachiasmatic nucleus and the parvocellular part of the PVN which include AVP-ir parvocellular neurons. Hybridization signals were also not found in the median eminence and the pars nervosa, the terminal regions of neurosecretory fibers.

Preparation of tissue sections and methodological checks

Among the fixatives tested, 4% PFA gave the most intense hybridization signals in the rat hypothalamus (Table 1). The use of a PGP

TABLE 1. Comparison of fixatives for *in situ* hybridization (ISH) of the AVP mRNA and immunohistochemistry (IHC) of AVP in the rat hypothalamus

Fixatives	Proteinase treatment	ISH	IHC	
			001[a]	1285[b]
Bouin's solution	Yes	+	+ +	+ +
Bouin's without acetic acid	Yes	+ +	NT	+ +
4% PFA	No	+ + + +	−	+ +
2% PFA+1% GLA	Yes	+ +	NT	NT
2% PFA+1% GLA+1% PA	Yes	+ + +	−	+
2% PFA+1% GLA+1% PA	No	+	+ + +	+ + +

PFA, paraformaldehyde; GLA, glutaraldehyde; PA, picric acid.
Note: −, not (or scarcely) stained; +, weakly stained; + +, moderately stained; + + +, strongly stained; + + + +, very strongly stained; NT, not tested.
[a] Anti-vasopressin antiserum (Bioproducts, batch #001).
[b] Anti-vasopressin antiserum (Bioproducts, batch #1285).

solution also yielded satisfactorily intense staining results, when tissue sections were treated with proteinase prior to incubation with labeled probes. Results of hybridization in frozen sections were similar to those in paraffin sections. These observations were consistent among the four oligo-nucleotide probes, while effects of fixation on immunohistochemical staining were rather complex, that is, stainabilities differed between the antisera utilized (Table 1). We are currently using the PGP solution with a proteinase treatment in the hybridization procedure.

The proteinase treatment of tissue sections fixed with the PGP solution markedly increased specific hybridization signals, although intensity of signals in 4 % PFA fixed sections was not increased by this treatment.

Acetylation seemed to prevent not only non-specific background binding of labeled probes, but also their specific base-pairing with the com-plementary nucleotide sequences. Thus, we did not adopt this treatment in our method. On the other hand, the increase in probe concentration above 1×10^4 cpm/μl markedly augmented unde-sirable background. The probe concentration of 5×10^3 cpm/μl gave clearly identifiable specific hybridization signals, although the signals were weak. These results indicate that the probe concentration around 1×10^4 cpm/μl may be appropriate for the oligonucleotide-mRNA ISH method for neurohypophysial hormones.

The distributional pattern and intensity of hy-bridization signals in paraffin sections stored for up to 18 months were similar to those in the initial sections which were hybridized immediately after being cut.

Specificity tests

RNase pretreatment   Hybridization signals in the magnocellular nuclei were almost completely diminished to the background level by the RNase pretreatment.

Tm   When the temperature of washing after hybridization with the AVT/OXT probe was raised to 50°C, hybridization signals were apparently reduced. Signals were further decreased along with elevation of washing temperature, and at about 65°C, almost all signals were removed. The

value of Tm estimated from the plot (Fig. 4) was about 51°C for pairing between the AVT/OXT probe and the OXT mRNA.

Absorption and competition tests   Addition of excess amounts of the synthetic template and the unlabeled probe to the hybridization mediums markedly reduced specific localization of silver grains. On the other hand, an excess amount of unlabeled mismatching probe did not change the localization and intensity of hybridization signals.

The use of alternate probes to the same mRNA   Hybridization signals of the AVP and AVP–NP probes were localized in the same areas in the SON and the PVN. When the unlabeled AVP probe was added to the labeled AVP–NP probe and *vice versa*, hybridization signals were not altered. Furthermore, the application of mixed AVP and AVP–NP probes conspicuously increased hybri-dization signals (Fig. 6), showing that the AVP and the AVP–NP probes may not interact each other.
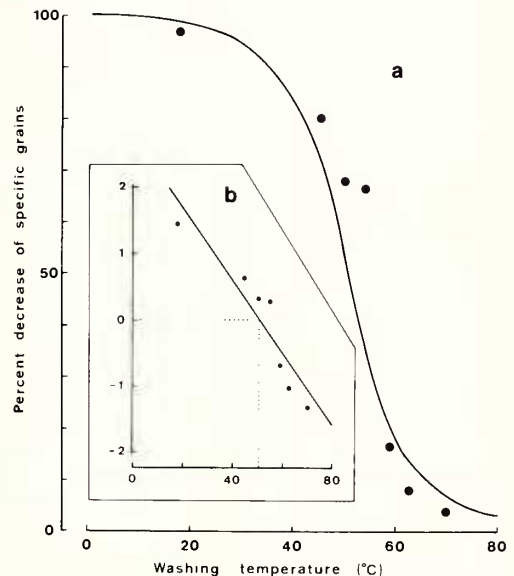
Cross species hybridization   In the magno-

FIG. 4.   Tm analysis for pairing of the AVT/OXT probe and the OXT mRNA by *in situ* hybridization. (a) Thermal denaturation of probe-mRNA duplexes. (b) Logit transformation and estimation of Tm value, showing that the value is about 51°C for pairing of the AVT/OXT probe and the OXT mRNA.
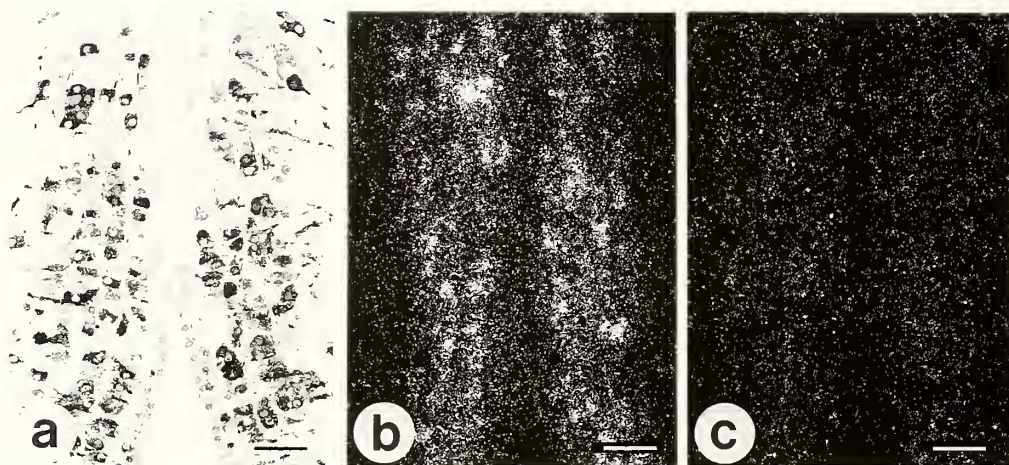
FIG. 5.   Immunoreactive (ir) AVT neurons (a) and hybridization signals of the AVT mRNA in the magnocellular
part of the toad preoptic nucleus (b, c). The AVT/OXT probe yielded intense hybridization signals (b), which
are distributed parallel to AVT-ir neurons (a). In contrast, the AVP probe yielded only weak hybridization
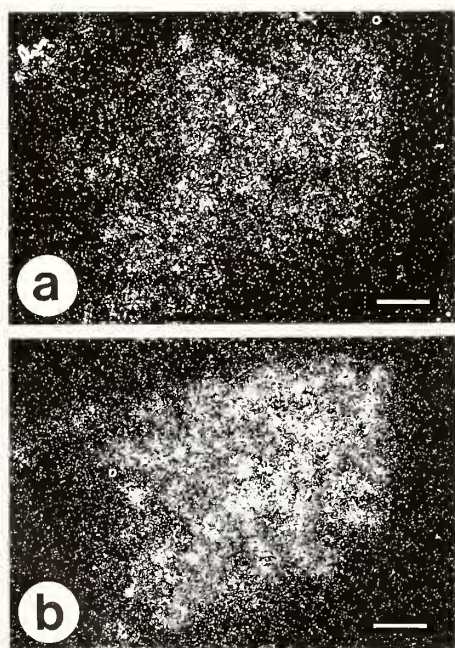signals (c). Scale bar, 50 μm.

FIG. 6.   In situ hybridization of the AVP mRNA with
the AVP probe only (a) and the mixture of AVP
and AVP–NP probes (b) in the paraventricular nu-
cleus. Note that the density of silver grains by the
mixed probe is higher than by the AVP probe only.
Scale bar, 50 μm.

cellular part of the toad preoptic nucleus, the
AVT/OXT probe yielded intense hybridization
signals, while those given by the AVP probe were
faint (Fig. 5). In contrast, in the rat hypothalamic
sections, intensity of hybridization signals induced
by the AVT/OXT probe was similar to that given
by the AVP probe. The signals by the AVT/OXT
probe were localized not only in the SON and the
PVN regions where ir-OXT neurons are predomi-
nant, but also in the regions occupied by ir-AVP
neurons with similar intensity to that seen in the
OXT regions. The same result was obtained in
another independent study on the hypothalamus of
the ICR strain mouse (unpublished).

*Distribution of ISH signals vs. that of AVP- and
OXT-immunoreactivity*   As is described above,
the distribution of hybridization signals was consist-
ent with the immunohistochemical localization of
related peptides. However, the intensity of hybri-
dization signals did not necessarily correlate with
that of immunoreactivity, as was reported pre-
viously [11]. Immunoreactive neurons were some-
times not labeled with the probe, and *vice versa*.

DISCUSSION

The present study showed that 22mer synthetic
oligonucleotides as probes for mRNAs of neurohy-

pophysial hormones were localized in the hypo-thalamic magnocellular neurosecretory nuclei in the toad and the rat after ISH stainings. The distributions of the probes were consistent with those of immunoreactivities to related peptides, e.g., the AVP probe was localized in the dorso-lateral region of the PVN and the ventral region of the SON where ir-AVP neurons are predominant. However, suprachiasmatic neurons in which Uhl and Reppert [6] demonstrated intense hybridiza-tion signals for the AVP mRNA did not show noticeable hybridization signals in our study. Since the intense signals in the suprachiasmatic nucleus have been reported only by Uhl and Reppert, we consider that the above discrepancy is due to longer autoradiographic exposure time by them, judging from their published photographs.

The disappearance of hybridization signals after the RNase pretreatment and the estimated Tm value indicate that the oligonucleotide probes were actually paired with tissue RNAs by hydrogen bonds. Other specificity tests showed that the present probes specifically recognize the com-plementary nucleotide sequences in particular mRNAs. The consistency of the distribution of hybridization signals for AVP and OXT mRNAs with those of AVP and OXT immunoreactivities further supports the occurrence of specific base pairings between the probes and the related tissue mRNAs. The discrepancy in the distribution of hybridization signals and immunoreactivities at the cellular level must be considered with information concerning secretory activity of neurosecretory neurons [11]. We thus convince that the AVP and AVP–NP probes were hybridized with the rat AVP mRNA, the OXT–NP probe paired with the rat OXT mRNA, and the AVT/OXT probe recognized the toad AVT mRNA and the rat OXT and AVP mRNAs.

The cross species hybridization study clarified that the 22mer oligonucleotide probes discrimi-nated nucleotide sequences which include mis-matching bases at more than 2 positions, although one-point mismatching was not recognized. This result strongly supports a reliability of the present ISH method in the study of mRNAs for neurohy-pophysial hormones. Further, it suggests that the oligonucleotide-mRNA ISH technique is widely applicable to studies of gene expressions for various peptides and proteinaceous hormones with high fidelity. The method may also be employable in detection of expressed genes concerning he-reditary diseases.

Our present study showed that, as to the hypothalamic magnocellular neurons, the distribu-tion of hybridization signals is coincide with that of immunohistochemical staining, indicating that the ISH method is sufficiently sensitive to study gene expression of neurohormones. Nonetheless, one of disadvantages in the ISH method using oligo-nucleotide probes is that labeling of multiple sites in a single probe molecule is rather difficult. The present result that an application of a mixture of the AVP and AVP–NP probes yielded a marked increase in specific signals suggests a solution for the above problem, since an interaction between the AVP and AVP–NP probes seems to be negligible. Thus, a use of mixed probes each of which recognized a different region in the same mRNA probably enhances hybridization signals, when an increase in the sensitivity of the oligo-nucleotide-mRNA ISH method is required.

REFERENCES

1 Ivell, R. and Richter, D. (1984) Structure and comparison of the oxytocin and vasopressin genes from rat. Proc. Natl. Acad. Sci. USA, **81**: 2006–2010.

2 Lathe, R. (1985) Synthetic oligonucleotide probes deduced from amino acid sequence data. Theoreti-cal and practical considerations. J. Mol. Biol., **183**: 1–12.

3 McCabe, J. T., Morrell, J. I., Ivell, R., Schmale, H., Richter, D. and Pfaff, D. W. (1986) *In situ* hybridization technique to localize rRNA and mRNA in mammalian neurons. J. Histochem. Cytochem., **34**: 45–50.

4 Sherman, T. G., McKelvy, J. F. and Watson, S. J. (1986) Vasopressin mRNA regulation in individual hypothalamic nuclei: a northern and *in situ* hybrid-ization analysis. J. Neurosci., **6**: 1685–1694.

5 Uhl, G. R., Zingg, H. H. and Habener, J. F. (1985)

Vasopressin mRNA *in situ* hybridization: localization and regulation studied with oligonucleotide cDNA probes in normal and Brattleboro rat hypothalamus. Proc. Natl. Acad. Sci. USA, **82**: 5555–5559.

6 Uhl, G. R. and Reppert, S. M. (1986) Suprachiasmatic nucleus vasopressin messenger RNA: circadian variation in normal and Brattlebore rats. Science, **232**: 390–393.

7 Wolfson, B., Manning, R. W., Davis, L. G., Arentzen, R. and Baldino, F., Jr. (1985) Co-localization of corticotropin releasing factor and vasopressin mRNA in neurons after adrenalectomy. Nature, **315**: 59–61.

8 Pavel, S. (1975) Vasotocin biosynthesis by neurohypophysial cells from human fetuses. Evidence for its ependymal origin. Neuroendocrinology, **19**: 150–159.

9 Pavel, S. (1980) Presence of relatively high concentrations of arginine vasotocin in the cerebrospinal fluid of newborns and infants. J. Clin. Endocrinol. Metab., **50**: 271–273.

10 Nojiri, H., Sato, M. and Urano, A. (1985) *In situ* hybridization of the vasopressin mRNA in the rat hypothalamus by use of a synthetic oligonucleotide probe. Neurosci. Lett., **58**: 101–105.

11 Nojiri, H., Sato, M. and Urano, A. (1986) Increase in the vasopressin mRNA level in the magnocellular neurosecretory neurons of water-deprived rats: *in situ* hybridization study with the use of synthetic oligonucleotide probe. Zool. Sci., **3**: 345–350.

12 Fujiwara, M., Hyodo, S., Sato, M. and Urano, A. (1985) Changes in vasopressin and oxytocin mRNA levels in the rat hypothalamus by oral hypertonic saline. Zool. Sci., **2**: 990 (Abstract).

13 Majzoub, J. A., Rich, A., van Boom, J. and Habener, J. F. (1983) Vasopressin and oxytocin mRNA regulation in the rat assessed by hybridization with synthetic oligonucleotides. J. Biol. Chem., **258**: 14061–14064.

14 Wallace, R. B., Shaffer, J., Murphy, R. F., Bonner, J., Hirose, T. and Itakura, K. (1979) Hybridization of synthetic oligodeoxyribonucleotides to Φ χ 174

DNA: the effect of single base pair mismatch. Nucleic Acids Res., **6**: 3543–3557.

15 Maxam, A. and Gilbert, W. (1980) Sequencing end-labeled DNA with base-specific chemical cleavages. Methods in Enzymology, **65**: 499–560.

16 Brahic, M. and Haase, A. T. (1978) Detection of viral sequences of low reiteration frequency by *in situ* hybridization. Proc. Natl. Acad. Sci. USA, **75**: 6125–6129.

17 Lawrence, J. B. and Singer, R. H. (1985) Quantitative analysis of *in situ* hybridization methods for the detection of actin gene expression. Nucleic Acids Res., **13**: 1777–1799.

18 Moench, T. R., Gendelman, H. E., Clements, J. E., Narayan, O. and Griffin, D. E. (1985) Efficiency of *in situ* hybridization as a function of probe size and fixation technique. J. Virol. Meth., **11**: 119–130.

19 Nojiri, H., Ishida, I., Miyashita, E., Sato, M., Urano, A. and Deguchi, T. (1987) Cloning and sequence analysis of cDNAs for neurohypophysial hormones vasotocin and mesotocin for the hypothalamus of toad, *Bufo japonicus*. Proc. Natl. Acad. Sci. USA, **84**: 3043–3046.

20 McBride, L. J. and Caruthers, M. H. (1983) An investigation of several deoxynucleoside phosphoramidites useful for synthesizing deoxyoligonucleotides. Tetrahedron Lett., **24**: 245–248.

21 Hayashi, S., Gillam, I. C., Delaney, A. D. and Tener, G. M. (1978) Acetylation of chromosome squashes of *Drosophila melanogaster* decreases the background in autoradiographs from hybridization with [^{125}I]-labeled RNA. J. Histochem. Cytochem., **26**: 677–679.

22 Kelsey, J. E., Watson, S. J., Burke, S., Akil, H. and Roberts, J. L. (1986) Characterization of proopiomelanocortin mRNA detected by *in situ* hybridization. J. Neurosci., **6**: 38–42.

23 Jokura, Y. and Urano, A. (1985) Projections of luteinizing hormone-releasing hormone and vasotocin fibers to the anterior part of the preoptic nucleus in the toad, *Bufo japonicus*. Gen. Comp. Endocrinol., **60**: 390–397.